Att #11/B

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re PATENT APPLICATION of

BRANSTROM et al.

Appln. No. 08/711,961 Group Art Unit: 1805

Filed: September 6, 1996 Examiner: J. Railey

Title: BACTERIAL DELIVERY SYSTEM

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DECLARATION UNDER 37 C.F.R. § 1.132

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

Sir:

I, Arthur A. Branstrom, a citizen of the United States of America, hereby declare and state as follows:

1. I hold a Ph.D. in Biomedical Sciences from Wright State University and have worked in the field of Molecular Biology for more than 10 years. I am a member of the American Society for Microbiology and currently hold the position of Senior Research Scientist at Transcell Technologies, Inc.

2. I am an Inventor on Patent Application No. 08/711,961, and am familiar with the prosecution history of the application, including the Office Action issued October 10, 1997.

3. In the patent application, a means for mutating bacteria by deletion of the *asd* gene within the DAP pathway is described. This mutation results in a strain of attenuated bacteria which is unable to synthesize components required for its cell wall. The consequence of this is that such bacteria

are able to enter target eukaryotic cells, but once inside the cells, the bacteria lyse and die, thereby delivering plasmid DNA to the cells.

4. In the application it is also disclosed that other methods of producing such mutated bacteria are known and would function according to the invention. Several examples are provided at page 9, lines 22-23 of the specification.

5. In the Office Action of October 10, 1997, claims 28-33 and 44 were rejected by the Examiner as not being enabled. It was the Examiner's position that the disclosure was enabling only for claims limited to *Shigella* strains that have been genetically attenuated by inactivation of the wild-type *asd* gene.

6. Attached hereto are a number of references which demonstrate that mutated bacteria according to the invention can be produced as disclosed and claimed in the present application by persons of skill in the art. The relatedness of the *E. coli* and *Shigella* genomes makes possible the extrapolation from the characterization of a given gene in one organism to its role in the other. Means of producing such attenuated bacteria include:

a. Bacterial autolysins (Genbank seq. #D17366)

b. Phage mediated lysis

c. Alteration of genes which affect the bacterium's ability to sythesize or acylate LPS (lipopolysaccharide)

d. Alteration of genes which affect synthesis of RNA and DNA;

e. Alteration of genes which degrade aberrant periplasmic proteins.

7. Bacterial Autolysins

Exploitation by overexpression of autolysins (for example, Genbank #D17366, others are also available), a system used by bacteria to degrade their cell walls in a controlled manner by using endogenously produced enzymes, once the bacterium is inside the host cell would lead to a delivery strain with the desired characteristics to function according to the invention.

8. Phage mediated lysis

For example, the publication of Steiner et al. (J. Bacteriology 175:1038-1042, 1993) demonstrates that it is possible to produce a mutated bacterium having the characteristics necessary for the present invention by construction of a bacterium (*E. coli*) with the genetic material encoding bacteriophage derived proteins capable of cell lysis. Construction of such bacteria can be carried out by persons of skill in the art using routine experimentation.

9. LPS synthesis or acylation

Genes that affect a bacterium's ability to synthesize or acylate LPS, such as *galU*, *rfe*, and *htrB*, can also be manipulated to produce an attenuated bacterium which will function according to the invention

In *Shigella*, mutations in LPS production result in attenuated strains that retain the ability to invade cells, but have lost their ability to spread from cell to cell, as shown by Sandlin et al. (Infection and Immunity 63:229-237, 1995). The gene sequences for *galU* can be found in the sequence databank GenBank for *E. coli* (accession no. M98830) and *Shigella flexneri* (accession no. L32811) *galU*.

htrB genes are essential to bacterial survival at temperatures greater than 33°C. As described in Karow et al. (J. Bacteriology 173:741-750, 1991), insertional inactivation of *htrB* leads to an arrest in cell division followed by the formation or bulges or filaments. Such cells would be particularly suited to the invention since they would grow *in vitro* at 30°C, and perform according to the invention *in vivo* at 37°C.

10. DNA and/or RNA synthesis

Genes that affect a bacterium's ability to synthesize DNA and/or RNA can also be manipulated to produce an attenuated bacterium which will function according to the invention.

Mutations in *thyA* have been shown to prevent the intracellular multiplication of *Shigella*, while not affecting its ability to invade the host cell (Yoshikawa et al, Vaccine 13:1436-1440, 1995). The complete nucleotide sequence of this gene is known, both for *E. coli* (accession no. J01710) and *Shigella* (accession no. S75211).

The lethality of a mutation is one of the best characteristics to exploit in the construction of delivery strains of the invention. Manipulation of the genes involved in DNA synthesis often has lethal effects to a bacterium. A mutation in *dut* (deoxyuridine triphosphatase) has been demonstrated in *E. coli* to result in the death of the organism. Inducible mutations of this type can be produced to trigger the death of the bacterium once inside the host cell. The sequence of the *dut* gene is known (accession no. AE000441). It would be a matter of routine experimentation for a researcher experienced in molecular biological techniques to generate inducible mutations within this gene or others using techniques known in the art.

11. Degradation of aberrant proteins

Mutations within *htrA* (an endopeptidase) have been constructed in *S. coli* and the *htrA* gene found to be indispensible for bacterial survival (Lipinska et al., J. Bacteriology 172:1791-1797, 1990). Mutations in *htrA* in *Salmonella typhimurium* resulted in strains that retained the ability to invade, but had diminished capacity to survive within macrophages and host tissues (Baumler et al. Infection and Immunity 62: 1623-1630, 1994; Johnson et al., Molecular Microbiology 5: 401-407, 1991). An *htrA* mutation in *Shigella* should result in a bacterium which is able to attach and invade, but will lyse and die within the cell.

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12. As the above examples demonstrate, numerous techniques are available such that an ordinarily skilled molecular biologist would be able through routine experimentation to constuct attenuated *Shigella* and other bacteria, as disclosed and claimed in the present application.

13. I declare further that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and such willful false statements may jeopardize the validity of the instant patent specification or any patent issuing thereon.

By Arthur A. Branstrom Date 01/07/98